

Fast photochemical oxidation of proteins (FPOP): A powerful mass spectrometry–based structural proteomics tool

Published, Papers in Press, July 1, 2019, DOI 10.1074/jbc.REV119.006218

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Edited by Wolfgang Peti

Fast photochemical oxidation of proteins (FPOP) is a MS-based method that has proved useful in studies of protein structures, interactions, conformations, and protein folding. The success of this method relies on the irreversible labeling of solvent-exposed amino acid side chains by hydroxyl radicals. FPOP generates these radicals through laser-induced photolysis of hydrogen peroxide. The data obtained provide residue-level resolution of protein structures and interactions on the microsecond timescale, enabling investigations of fast processes such as protein folding and weak protein–protein interactions. An extensive comparison between FPOP and other footprinting techniques gives insight on their complementarity as well as the robustness of FPOP to provide unique structural information once unattainable. The versatility of this method is evidenced by both the heterogeneity of samples that can be analyzed by FPOP and the myriad of applications for which the method has been successfully used: from proteins of varying size to intact cells. This review discusses the wide applications of this technique and highlights its high potential. Applications including, but not limited to, protein folding, membrane proteins, structure elucidation, and epitope mapping are showcased. Furthermore, the use of FPOP has been extended to probing proteins in cells and *in vivo*. These promising developments are also presented herein.

A fundamental understanding of how a given protein functions generally requires a detailed characterization of its underlying structure and dynamics. This entails a comprehensive description of a protein's conformation and interactions. In recent years, mass spectrometry (MS)-based methods have been increasingly used to study protein structure. Methods such as native MS, ion-mobility spectrometry, chemical cross-linking, and others have been used to study a wide variety of protein systems, including membrane proteins, in various levels of complexity, including *in vitro*, in cells, in tissue, and *in vivo* (1–7). These methods, which have been reviewed elsewhere (8), fill a gap in analysis of proteins that are difficult to study by crystallography and NMR. Although these methods cannot provide atomic-level resolution, the use of MS as the

analytical readout has several advantages, including the need for only microgram quantities of protein as well as the ability to study large proteins and complex samples.

Protein footprinting methods are another constituent of the MS-based structural biology toolbox. These methods investigate structure and interactions via the covalent labeling of proteins. Liquid chromatography coupled to high-resolution MS (LC-MS/MS) is used to identify modified amino acids and quantify the extent of labeling. Since the rise of hydrogen deuterium exchange coupled to MS (HDX-MS)² in the 1990s (9), MS-based footprinting methods have been increasingly used for analysis of higher-order structure. In most cases, footprinting reports on the solvent accessibility of amino acid side chains, which is altered upon ligand binding or changes in conformation. The lone exception is HDX-MS, where alterations in the hydrogen bonding network on the backbone are required for labeling (10, 11). Coupling of these footprinting methods with bottom-up proteomics, where proteins are proteolyzed and the resulting peptides are analyzed by MS, results in localized information on interaction sites and regions of conformational change. In some cases, residue-level resolution can be achieved providing higher-resolution structural information (12–14). Protein footprinting methods have been successfully used to probe higher-order structure of large proteins such as antibodies (15–17) and large assemblies (18). In addition, these methods have been used to study complex systems such as membrane proteins in detergents (19, 20), micelles (21), nanodiscs (22), viruses (23), and intact cells (24, 25).

One type of footprinting method, hydroxyl radical protein footprinting (HRPF), utilizes hydroxyl (OH) radicals to oxidatively modify the side chains of amino acids. This irreversible labeling method can modify 19 of 20 amino acids making it a general labeling strategy (26). Although modifications of +16 Da dominate the HRPF data, there are many other modifications that amino acids can undergo, including the addition of a carbonyl group (+14 Da) on several mostly hydrophobic amino acids and decarboxylation (–30 Da) of the carboxylic acids. The various modification types by HRPF (Table 1) and the chemistry have been reviewed extensively elsewhere (27). Hydroxyl

This work was supported by National Institutes of Health Grant R01 GM128985 and startup funds from the University of Maryland. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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² The abbreviations used are: HDX-MS, hydrogen deuterium exchange coupled to MS; FPOP, fast-photochemical oxidation of protein; RMSD, root-mean-squared deviation; HRPF, hydroxyl radical protein footprinting; NEM, *N*-ethylmaleimide; GEE, glycine ethyl ester; DEPC, diethylpyrocarbonate; OH, hydroxyl; A1AT, α_1 -antitrypsin; MP, membrane protein; CFTR, cystic fibrosis transmembrane conductance regulator; MM, molecular modeling; SASA, solvent-accessible surface area; IC-FPOP, in-cell FPOP; IV-FPOP, *in vivo* FPOP; BR, bacteriorhodopsin.

Table 1**Mass changes of amino acids modified by HRP with amino acids listed in order of hydroxyl radical reactivity rates**

Side chain	Mass change
Cys	+48, +32, -16
Met	+16, +32, -32
Trp	+16, +32, +48, -32
Tyr	+16, +32
Phe	+16, +32
His	+16, -23, -22, -10, +5
Leu	+16, +14
Ile	+16, +14
Val	+16, +14
Pro	+16, +14
Arg	+16, +14, -43
Lys	+16, +14
Glu	+16, +14, -30, -44
Gln	+16, +14
Asp	+16, -30, -44
Asn	+16
Ser	+16, -2
Thr	+16, -2
Ala	+16

radical-based footprinting has been traditionally used for nucleic acid footprinting. The seminal work by Tullius and Dombroski (28) used hydroxyl radicals to map the protein interaction sites of DNA. The method is still used for this application as well as for mapping the tertiary structure of RNA (29). The method was first coupled with MS and applied for protein footprinting by Chance and co-workers (30–32), who have demonstrated its use for mapping protein structure. There are multiple means to generate hydroxyl radicals for labeling, including Fenton chemistry (33), radiolysis of water (34), and electrochemistry (35, 36). These methods label proteins on the millisecond to second timescale. This laser-based method generates hydroxyl radicals via photolysis of hydrogen peroxide (H_2O_2) labeling proteins on the nanosecond to microsecond timescale, allowing for the study of interactions with fast off rates (37, 38). This review will focus on the laser-based HRP method of fast photochemical oxidation of proteins (FPOP).

FPOP: a laser-based HRP method

In FPOP, a flowing solution of protein and H_2O_2 is irradiated by an excimer laser at 248 nm to generate hydroxyl radicals (Fig. 1) (38, 39). The laser beam is then focused with convex lenses on a 150–450- μm inner diameter silica tubing that is used as the flow tube (40). The sample is irradiated through a transparent window exposed on the coated silica tubing. The flow rate and laser frequency are coordinated so that each protein molecule is only irradiated once. After irradiation, the sample is collected into a tube containing catalase and free methionine in buffer to quench H_2O_2 and OH, respectively, thus preventing post-footprinting oxidation artifacts from any remaining reactive species. To correct for the background oxidation, protein control samples are introduced into the flow system without laser irradiation (40). Performing FPOP under constant flow limits over-oxidation, which could lead to protein unfolding. A radical scavenger, most commonly glutamine, is also added to the sample as another experimental control to prevent over-oxidation. Based on the reactivity of glutamine with OH, FPOP labels proteins on the microsecond timescale and ensures labeling of the native state of proteins (41, 42).

FPOP has the ability to report protein transient dynamics, including fast folding and alterations in side-chain flexibility, and fast fluctuations upon ligand binding. It has been successfully used to identify protein interactions sites and regions of conformational change (43–45). Protein systems studied by FPOP vary, including heterogeneity in protein size and sample complexity. Applications are broad, like epitope mapping (12, 46), identifying lipid-interacting regions in membrane proteins (22), and the structural reorganization of viral proteins (45). FPOP has also been applied to the study of intact cells providing structural information across the proteome (24, 47). Recently, the use of FPOP-derived data has been implemented in molecular modeling studies demonstrating its potential for *de novo* modeling of protein structure (48, 49). These results demonstrate the efficacy of the method for a variety of applications and for studying a wide variety of proteins. In this review, we will highlight the varied applications of FPOP and new developments in the field.

Comparison of protein footprinting methods used to study higher-order protein structure

Regarding footprinting, there are several methods that are currently being used in protein structure studies. They vary in terms of specificity, reversibility, and timescale of labeling (Table 2). These differences provide advantages based on the type of protein system that is being studied. Specific labels such as *N*-ethylmaleimide (NEM), which specifically labels cysteines, and glycine ethyl ester (GEE), which labels carboxylic acids, have a significantly reduced complexity in data analysis because only one or two amino acid types can be modified. Many cysteines are critical for redox processes, and their activity strongly depends on their oxidative status (50). NEM has been successfully used to identify such cysteine residues (51, 52). GEE modifies carboxylic acids through a 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-mediated coupling reaction (53). This reaction can lead to relatively rapid and quantitative modification of solvent-accessible carboxyl groups under mild conditions. Aspartate and glutamate side chains, often located on the surface of proteins, play important roles in electrostatic interactions and are essential for enzymatic activities. Because of this, the GEE coupling reaction has been successfully used in probing the enzymatic activity of several proteins (54–56). However, the high specificity of these labeling methods limits their use to proteins that both contain these specific amino acids and where these amino acids are involved in the structural/interaction changes these proteins undergo. In addition, the slow timescale of labeling reduces their use for weak interacting systems with fast off rates or fast folding events.

Diethylpyrocarbonate (DEPC), a less specific footprinting method, labels all nucleophilic residues (57) and can probe up to 30% of the residues in the average protein. These include His, Lys, Tyr, Ser, Thr, and Cys residues (58) increasing the use of the method for a wider variety of proteins. The increased number of modified residues does increase the complexity of data analysis but not as extensively as general labeling strategies. One limitation of this labeling technique is cysteine scrambling. After labeling, cysteine bonds are typically reduced prior to MS analysis. This alleviates cysteine residues, which can take the

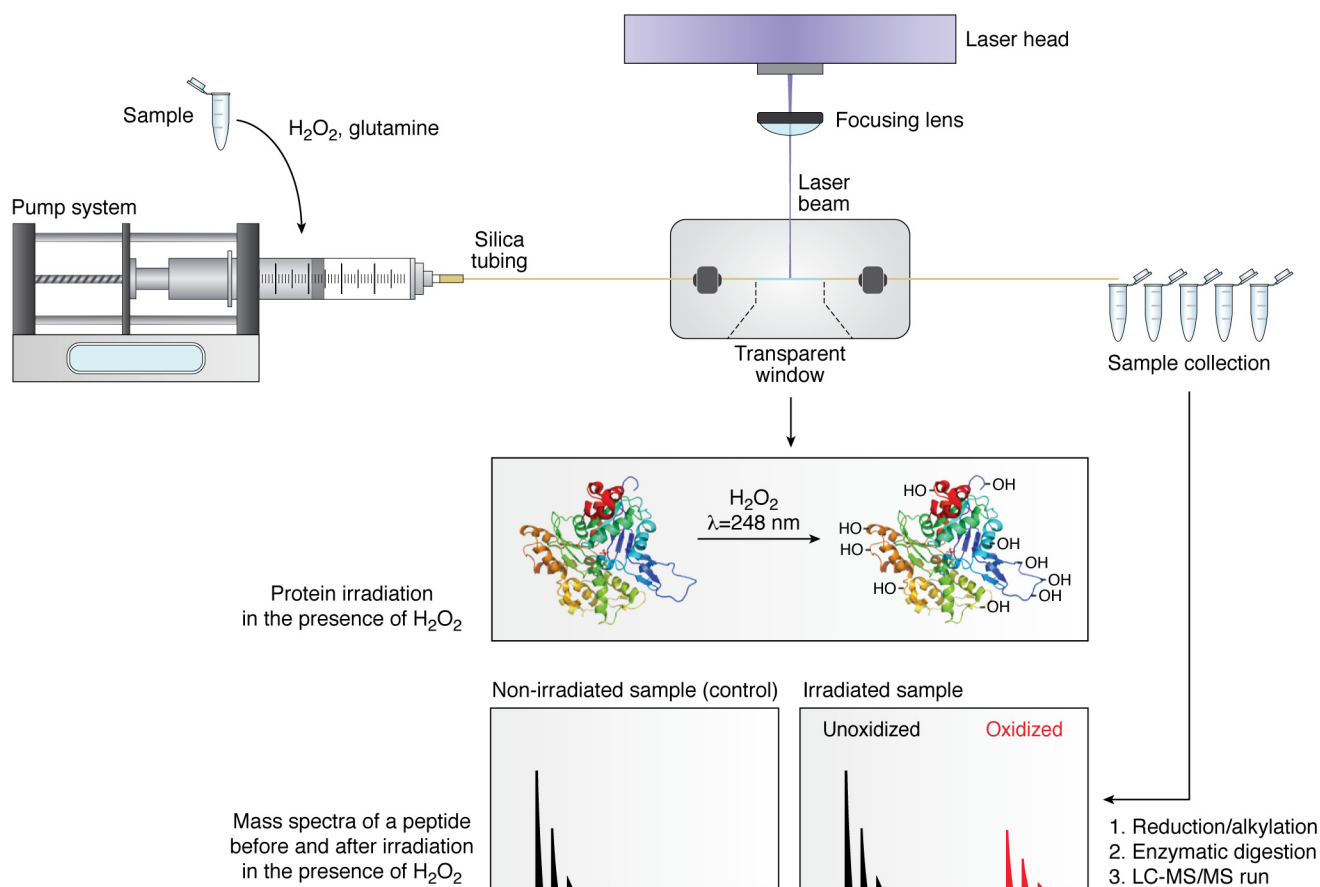


Figure 1. Scheme of a typical *in vitro* FPOP setup.

label from an originally labeled residue, leading to misinterpretation of the results (58).

In contrast to these methods, HDX-MS and HRPf are general labeling strategies that can both label multiple amino acids. HDX-MS has been by far the most widely used method to study protein structure, dynamics as well as protein–protein, protein–macromolecule, and protein–ligand interactions. This method reflects both solvent accessibility and hydrogen bond network changes with backbone amide hydrogens being interrogated. Under typical experimental conditions, all amino acids except proline, which does not have an amide hydrogen group, can be labeled by this method, and all amide hydrogens can be expected to exchange if not protected by secondary, tertiary, or quaternary structure. Furthermore, continuous labeling experiments have allowed for probing of conformational dynamics and structural changes in a time-resolved manner, whether slow and cooperative (EX1) or fast and stochastic (EX2) (59–61). However, the reversible nature of the HDX label is a limitation, and careful experimental conditions have to be used to limit back exchange prior to MS analysis. Primarily, HDX-MS is not amenable to the post-labeling sample handling approaches that are required for proteome-wide studies and therefore remains most powerful as an *in vitro* method.

An advantage of the HRPf label is its irreversibility, which allows for a more flexible post-labeling sample, such as handling the use of longer chromatographic gradients and the use of enrichment strategies for complex samples. Another advantage is the microsecond timescale of FPOP labeling, which is

faster than proteins can unfold. Because of the time-scale difference between FPOP and HDX-MS, FPOP helped identify an allosteric conformational change that occurred in the loop region of thrombin, which was not detected by HDX-MS (12). Like HDX-MS, FPOP has also been utilized for higher-order structure characterization and is rapidly gaining a reputation as a complementary approach that combines the possibility of obtaining information on the residue level and a time resolution that lies within the timescale of early protein folding events. The high speed and irreversible character of its labeling makes FPOP-MS a very powerful tool in the study of the folding and unfolding events of proteins (62, 63). For example, in 2012, Stocks *et al.* (64) published a study on the folding process of α_1 -antitrypsin (A1AT), which provided valuable data on the A1AT folding process. An interesting example of FPOP experiments performed on the sub-second scale with amino acid resolution was presented in two different articles by Gross and co-workers (65, 66). These studies follow the barstar protein-folding dynamics, which unfolds at 0 °C and folds with a temperature jump (*T*-jump) (67, 68), which is achieved by the consecutive irradiation of two different lasers. The delay between the two laser pulses is varied to obtain different time points (Fig. 2A). The amount of solvent-accessible residues decreases as the protein folds and so does the amount of modifications observed after FPOP (Fig. 2B).

Although FPOP has been successfully used for structural characterization, it comes with its share of pitfalls such as the use of specific buffers that must be compatible with OH (*i.e.*

Table 2
Comparison of protein footprinting methods

	HDX	GEE	DEPC	NEM	FPOP
Labeling time scale	Seconds to hours	Seconds to minutes	Minutes	Minutes to hours	Microseconds
Type of label	Reversible	Reversible	Reversible for most residues Irreversible for Lys	Irreversible	Irreversible
Sensitivity/specificity	Backbone amide groups	Carboxylic acids	Nucleophilic residues, N terminus, His, Lys, Tyr, Ser, Thr, and Cys	Cys	19 out of 20 amino acids (excluding Gly)
Chemicals/materials	Commonly used buffers under physiological conditions; Quench solutions pH 2.5 (strong or weak acid) 0 °C; Denaturing (urea, guanidine HCl); Reducing agents (TCEP); Pepsin digestion	EDC stock solution prepared freshly in PBS buffer; MES/NaOH (pH 5.5); PBS (pH 6, 6.5, 7, 7.5); HEPES (pH 8, 8.5)	DEPC in acetonitrile in a molar excess of 4; 10 mM imidazole added to quench the reaction	NEM solution 10 mM HEPES, 5% DMSO	Various buffer solutions based on protein; H ₂ O ₂ , free glutamine radical scavenger Quench solution: free methionine and catalase (<i>in vitro</i>)
Limitations	Reversibility of the label limits its use for complex samples that require longer separation times	Carboxylic acid-specific labeling limits the proteins that can be studied	Free cysteine scrambling	Cysteine-specific labeling limits the proteins that can be studied	Residue-reactivity differences, complex data analysis, and expense of excimer laser

does not scavenge the radical), extensive post-labeling separation, and quantitation during analysis. Also, the cost of an excimer laser for H₂O₂ photolysis can be prohibitive for many labs. Another limitation of the method is that different amino acids have different reactivities with hydroxyl radicals (27). Amino acids are listed in order of reactivity in Table 1 with the most reactive residue, cysteine, at the top. Because of these differences, highly-reactive residues such as methionine may be modified whether they are solvent-accessible or not. Because the reactivity rates of the amino acids are known, it is possible to account for these rate differences to calculate a protection factor (49, 69). Furthermore, analysis of FPOP data are the most complex of the footprinting methods. Although there have been advances in analysis software (70–73) and quantitation methods (74), the field lags behind HDX-MS where both automated systems (75) and robust analysis software (76, 77) are available. Nonetheless, this technique has proved successful in many broad applications that support its versatility.

Footprinting methods can be used together in a complementary approach to gain increased structural information. For example, by using HDX-MS, FPOP, alanine shave mutagenesis (*i.e.* mutating potential key residues into alanine to study their effect on protein structure and functionality), and binding analytics in tandem, Li *et al.* (78) reported the identification of an energetic epitope by determining the interfacial hot spot that dominates the binding affinity for an anti-interleukin-23 (anti-IL-23) antibody (Fig. 3). Footprinting results show an overlap of important epitope regions detected by HDX-MS and FPOP. These results demonstrate FPOP and HDX-MS are equally useful for epitope structural mapping (78). In additional studies, FPOP, HDX-MS, and GEE labeling were used in tandem to demonstrate that the critical binding epitope of the IL-6/IL-R complex is the short segment ¹³⁵QNSPAED¹⁴¹. This integrated approach shows great utility for characterizing proteins and their complexes and can be applied to assist in optimizing the design of protein therapeutics (79).

The outlined advantages of FPOP make it capable of standing alone for many applications. The following sections are focused on how this unique footprinting method was gradually introduced into areas once lacking detailed structural information.

Applications: FPOP to study membrane proteins

Cellular membranes contain a large number of proteins representing ~30% of the total proteome (80). Membrane proteins (MP) are involved in crucial cellular functions, including respiration and signal transduction. MPs are also quite dynamic and flexible enabling them to perform different tasks with high efficiency. However, this makes structure determination challenging (22). Therefore, structural studies have been limited, especially for those proteins with high molecular weights and multidomain features (81). Until recently, X-ray crystallography, HDX, and NMR among others have been used by structural biologists to make seminal contributions (82). Where those techniques were lacking, cryo-electron microscopy (cryo-EM) picked up the slack to gain structural information of MPs (83).

MS-based methods are being increasingly applied in structural studies of MPs. Approaches used to study MPs by struc-

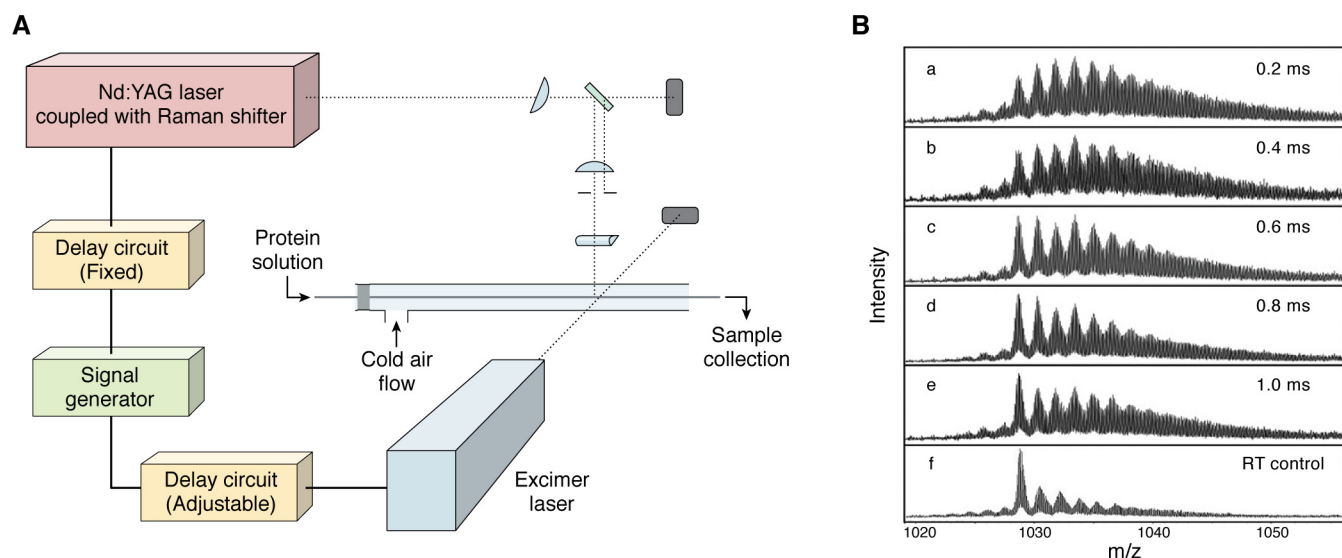


Figure 2. A, schematic of the flow system intersected by two laser beams at a window in the tube, as described previously for FPOP. The time between the two laser pulses is adjustable with the “delay circuit.” B, panels a–e, representative mass spectra of the barstar post-FPOP as a function of the time between the heating pulse and the FPOP probe. Panel f, mass spectrum of the barstar post-FPOP at room temperature as a control (70). Adapted with permission from Chen *et al.* (65).

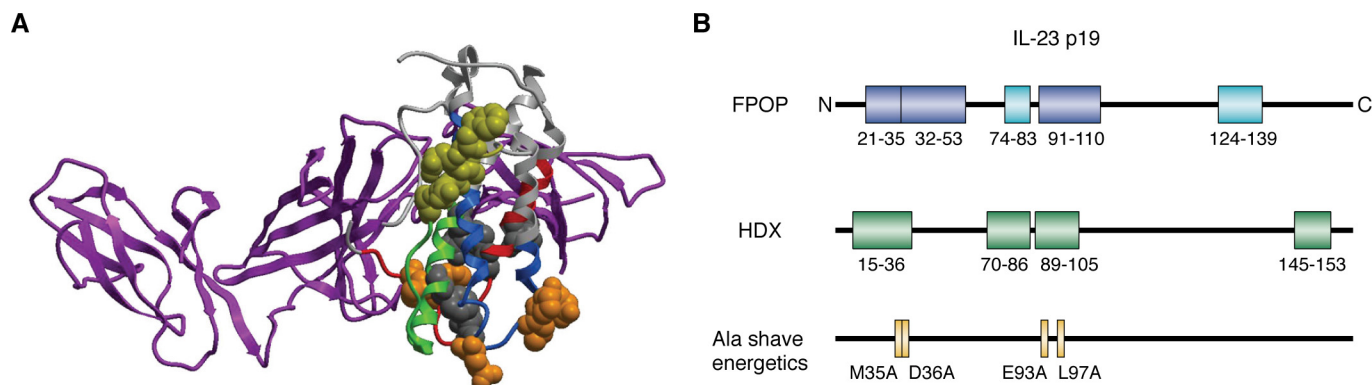


Figure 3. A, epitope regions determined by FPOP mapped on the crystal structure of IL-23. The p40 domain is shown in purple. The p19 domain is shown in grey. Region A is shown in red, region B is shown in blue, region C is shown in green. M5 residues are shown in orange. M6 residues are shown in dark grey. M7 residues are shown in yellow. B, epitope regions determined by FPOP, HDX, and alanine shave mutagenesis as mapped on the linear sequence of the IL-23 p19. Adapted with permission from Li *et al.* (78).

tural MS have been developed from those utilized to study water-soluble proteins, but the methods have had to be refined to overcome challenges unique to MPs such as intrinsic disorder, low abundance, heterogeneity, and transiency, but especially the requirement for solubilization by detergent micelles, membrane bilayers, or other amphiphiles (83). FPOP followed by MS detection and quantification is an excellent tool for these studies and can be used to provide information about structures, protein complexes, and conformational changes in solution. FPOP is particularly useful for the study of MPs in different amphiphiles. The irreversibility of its label allows the sample to be processed and to remove the lipids and detergents that are used to mimic the cell membrane, but are disadvantageous for LC-MS analysis (83). A pioneering study by Pan *et al.* (84) first demonstrated that FPOP could provide information on MP structure within a natural lipid environment. They carried out FPOP labeling of bacteriorhodopsin (bR), a methionine-rich MP. The advantage of studying this protein using FPOP is that it is highly susceptible to oxidative modifications due to methionine's faster reaction rate with hydroxyl radicals

compared with other amino acids (27). This study, together with a study carried out by Reading (85) (Fig. 4), determined that methionine oxidation occurred at a higher extent at solvent-accessible sites in bR (Met-32, -68, and -163) but not in membrane-protected regions (Met-20, -56, -60, -118, -145, and -209), supporting FPOP's capacity to study MPs.

Because detergents are a poor mimic of the native bilayer, a variety of detergent-free methods has been developed to solubilize MPs for biophysical analyses, namely lipid–protein nanodiscs that provide a better mimic of a native environment but with controllable stoichiometry of target MP. Lu *et al.* (22) studied these systems by inserting the light-harvesting complex 2 (LH2) from *Rhodobacter sphaeroides* into nanodiscs and, with the use of FPOP, validated the protein was still housed in a near-native state. Their results suggest a protein's outer membrane regions are more heavily labeled by hydroxyl radicals while the regions spanning the lipid bilayer remain protected. Studying proteins in nanodiscs with FPOP is a practical tactic to map extra-membrane protein surfaces and elucidate intrinsic MP topology (22). A different approach that can be used is

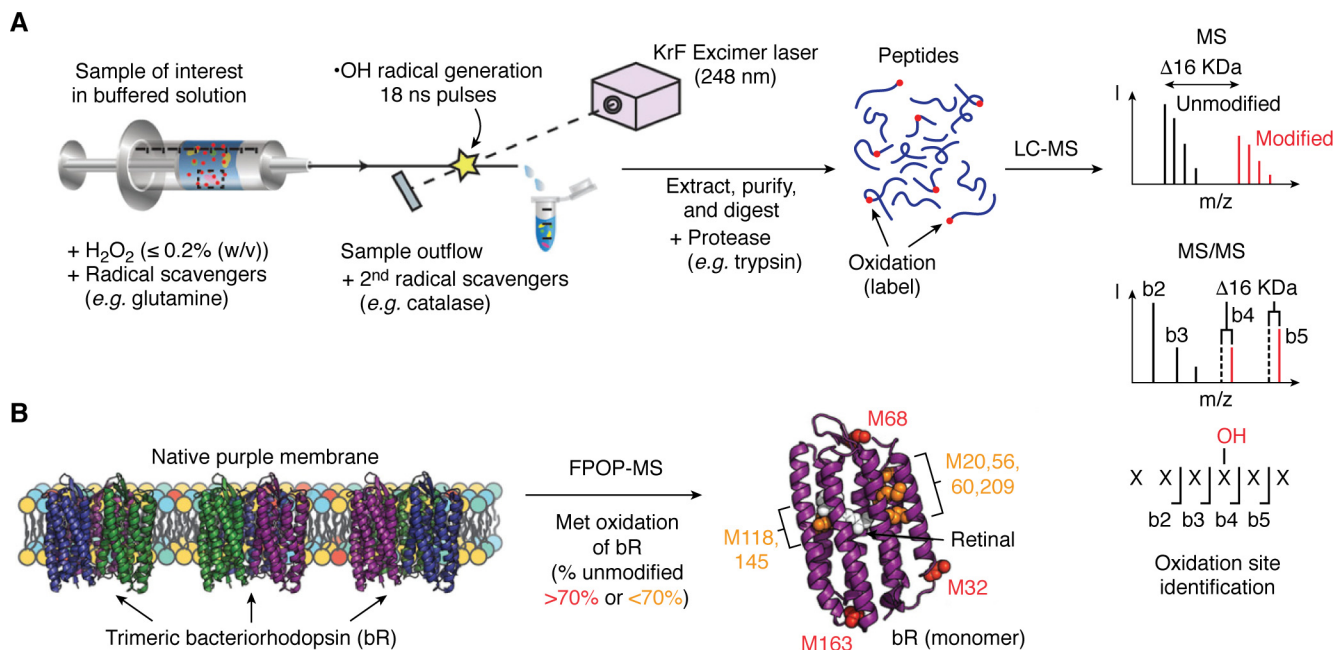


Figure 4. Footprinting MS of a membrane protein within a native environment using the FPOP method. A, typical workflow for membrane protein FPOP-MS within a native cellular environment. B, FPOP-MS of bR within its native purple membrane revealed that the extent of methionine (Met/M) residue oxidation correlates with the solvent accessibility and topology of the native bacteriorhodopsin structure. Adapted with permission from Reading (85).

reversed FPOP-MS, which solely measures the remaining unoxidized peptides because oxidized peptides often represent only a percentage of the oxidation product pool. The advantages of using this strategy include the simplification of data analysis due to this subtractive quantitation strategy as well as its capability to detect low-abundance peptides within membrane complexes. Using this approach, Yao and co-workers (81) examined nine peptides of cystic fibrosis transmembrane conductance regulator (CFTR) protein within the saponin semipermeabilized baby hamster kidney cell membranes (BHK-wtCFTR). CFTR mutants as the fundamental molecular defects of cystic fibrosis are currently the primary targets for the development of modulating drugs. They determined “structural marker” peptides that potentially report on the presence of two channel populations (open and closed) of CFTR within its native plasma membrane (81).

Significant advances have been made in the study of MPs by structural MS methods. Rapid developments in instrumentation and methodologies to date guarantee that MS will remain an integral component of the structural biology toolkit and show how MPs perform the vast array of functions and interactions essential for life. The systems described above are tunable and provide environments analogous to cells, but they may lack other characteristics of a cellular biological membrane such as cellular crowding, chaperones, and their local cellular pH.

Recent developments: modeling protein structures with the aid of FPOP

It is undeniable that the elucidation of proteins and protein complexes structures would not be feasible without the development of molecular modeling (MM). However, contrary to the methods used in the study of small organic molecules, MM calculations on proteins cannot always be used on their own due to the complexity of these systems, and so there is a need to

introduce experimental data in the calculations to obtain accurate results. This information is often taken from CD (86), NMR (87), HDX (88), and X-ray crystallography (89) experiments, among others. However, in recent years, FPOP-MS has provided valuable structural data, which was introduced in MM calculations furthering the depth of biological interpretation achieved from FPOP.

In one instance, Poor *et al.* (45) mapped the folding events of the paramyxovirus fusion protein, a crucial protein in the infection mechanism of the Paramyxoviridae family. They did so by using the atomic structures of pre- and postfusion states of a variant of the F protein made with an AMBER platform. For this large and highly hydrophobic protein, FPOP provided higher-resolution dynamic structural information that could be obtained with NMR or HDX-MS due to the size and glycosylation of the protein, making FPOP an interesting tool for the broader scientific community.

In 2015, FPOP data were first used as restraints in MD simulations to determine the structure of an early folding intermediate of barstar by Heinkel and Gsponer (90). They used FPOP restraints to back-calculate experimental ϕ values. These values are related to changes in the free energy of denaturation and account for the total native bonds for every residue environment, with $\phi = 1$ corresponding to the native structure. Therefore, they analyzed whether ϕ values and FPOP data could be used together to generate a structure where FPOP would improve the structural description of a folding intermediate state. Structures obtained using ϕ values exclusively were too compact and in poor agreement with the experimental β -Tanford value, which is an index of the compactness of the structures. The reason behind this effect is that ϕ values are calculated based on the amount of native contacts present in the structure only, allowing for nonnative contacts to form arti-

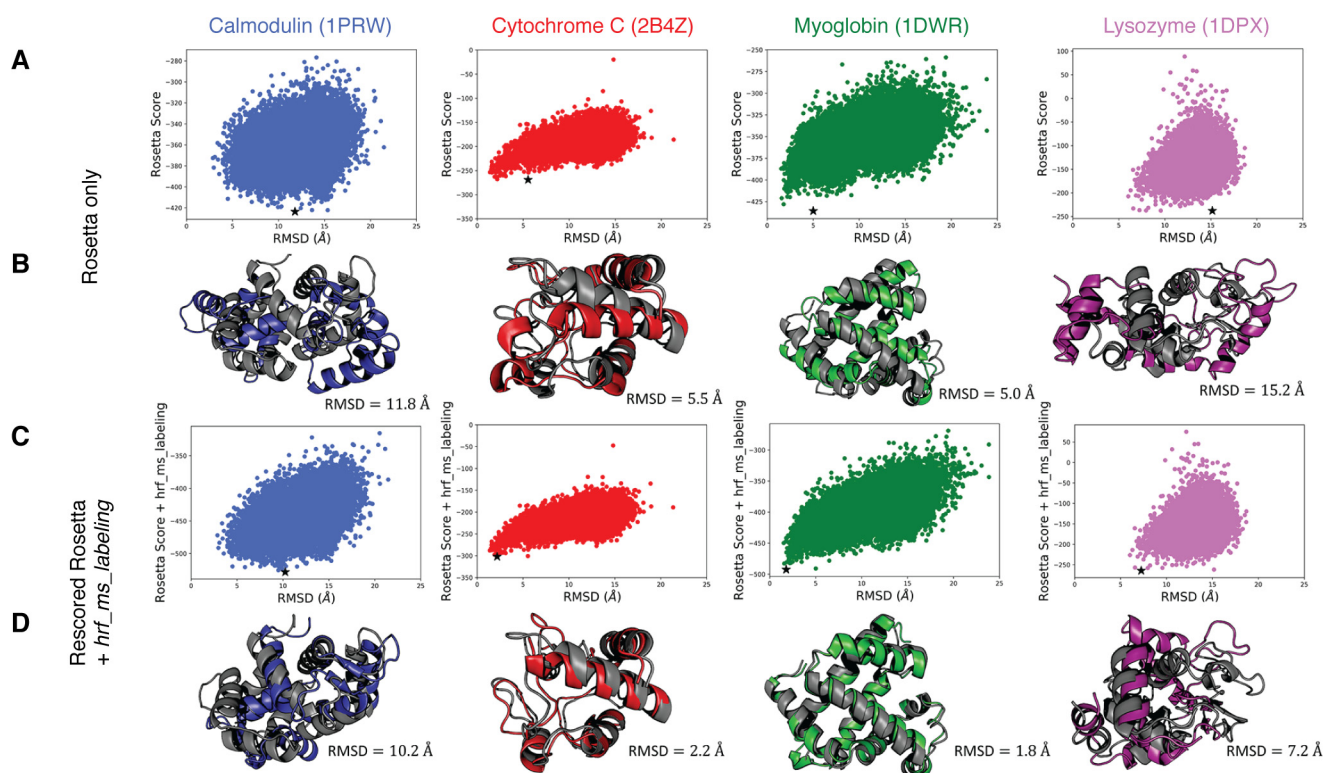


Figure 5. A, Rosetta score versus RMSD to the native structure plots for 20,000 models generated using Rosetta *ab initio* for each of the four benchmark proteins. The top-scoring model is represented as a star on each plot. B, top-scoring models from the Rosetta score versus RMSD distributions in A (color) superimposed on the respective native model (gray). C, Rosetta score + hrf_ms_labeling versus RMSD to the native structure plots for each of the four benchmark proteins after rescoring with the new score term. The top-scoring model is represented as a star on each plot. D, top-scoring models from the Rosetta score + hrf_ms_labeling rescoring distributions in C (color) superimposed on the respective native model (gray). Reprinted from Ref. 48.

cially on structures with a ϕ value smaller than 1, which will tend to conserve the compactness. In contrast, FPOP restraints are calculated with respect to a solvent-accessible surface area (SASA) and thus disfavor any type of interaction. Therefore, FPOP and ϕ data combined gave structures that are in much better agreement with the experimental β -Tanford values for barstar.

FPOP data also proved to be helpful on the determination of the binding site between a model heparin fragment and an HIV antigen using computational docking (91). This study carried out by Misra *et al.* (91) represents a step forward toward the exploration of new therapeutic avenues to stop or reduce HIV infection. More recently, Xie *et al.* (49) developed a workflow that successfully demonstrated a correlation between experimental FPOP data and calculated SASA values. As explained in their study, to convert high-resolution HRPD data into qualitative measurements of protein topography, there are four factors to take into account: 1) apparent oxidation rates must be accurately measured at the amino acid level; 2) oxidation must be normalized by the radical concentration and scavenging properties of the solution; 3) the inherent reactivity of the different amino acids must be accounted for; and 4) a quantitative relationship between normalized amino acid reactivity and SASA must be established. This workflow proved to be able to differentiate between low and high root-mean-squared deviation (RMSD) models, which makes it a useful validation tool for MM.

In a recent study, Aprahamian *et al.* (48) compiled a set of four soluble proteins with known crystal structures (calmodu-

lin, myoglobin, lysozyme, and cytochrome *c*) and generated a decoy set of 20,000 structures for each protein, which served as a benchmark to compare the structure prediction capabilities of Rosetta in the absence of FPOP-labeling data. The generated structures were scored using the Rosetta energy function, and scores versus RMSD to the native protein were generated (Fig. 5). Protein models were then rescored by adding a term to Rosetta's function that was developed to introduce FPOP labeling information. As a result, new top-scoring structures were obtained, which present smaller values of RMSD, including near-atomic resolution models for myoglobin and cytochrome *c*. This is the first method to incorporate experimental HRF/FPOP-labeling data in protein structure prediction. These studies demonstrate the potential of FPOP as a valuable method for the development of more accurate MM predictions with a broad application field.

Outlook: development of new footprinting methods and in vivo footprinting

The expansion of FPOP in the last decade has caught the attention of a broad community of scientists due to its relative simplicity. The versatility of this technique has led to the exploration of more complex biological systems previously out of its regime. Improvements to the current platform and conditions are being implemented to integrate FPOP to a broader number of studies. For instance, change in the oxidizing reagent might be beneficial for some applications. Although OH radicals have proved to be an excellent reagent to perform FPOP, there is an extensive universe of precursor molecules that produce radicals

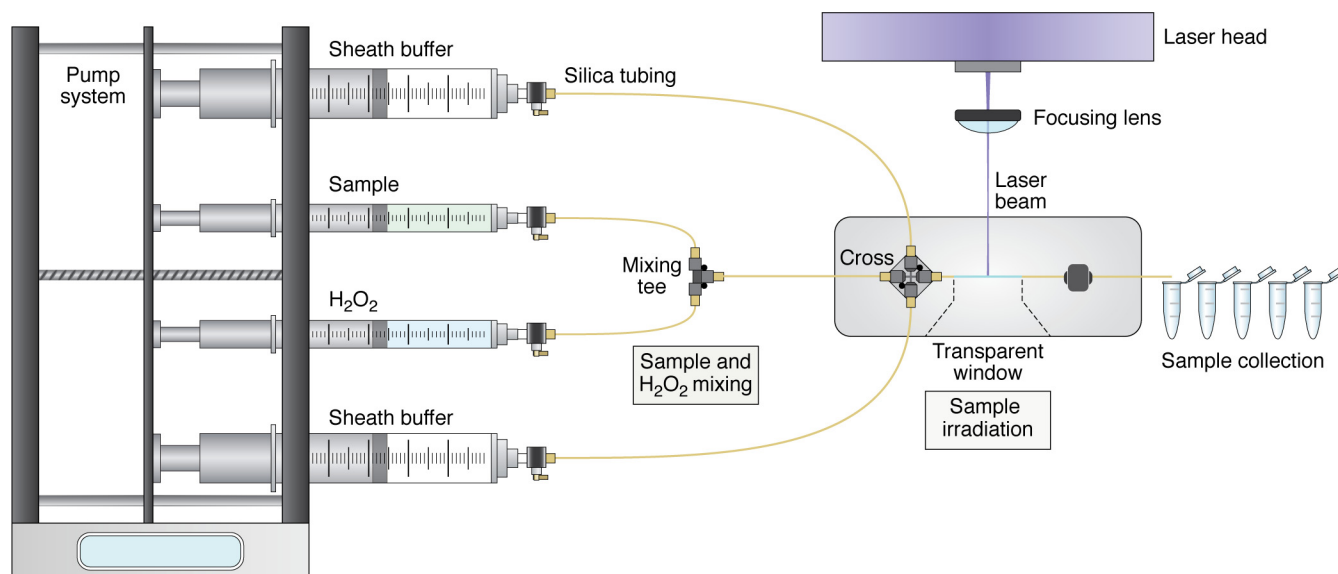


Figure 6. Scheme of a typical *in-cell* FPOP setup.

after irradiation followed by homolytic dissociation, which augurs great possibilities for future development of footprinting on the FPOP platform. The efficiency of different footprinting species like sulfate radical anion (92), iodine radical (93), carbenes (94), and trifluoromethyl radicals (95) were also introduced on a FPOP platform, projecting them as interesting candidates for protein-structure studies. Trifluoromethylation presents some further advantages because it can label amino acids embedded in membranes, and modifications can be detected not only by MS but also by ^{19}F NMR. The development of new reagents for use on the FPOP platform will further broaden the application of laser-based footprinting.

One factor that may limit the widespread adoption of FPOP is the treatment of the data. Because there are many factors that scavenge the radical prior to protein labeling, including metal ion chelators and reducing agents, a measure of a radical dose in each experiment would be beneficial. Dosimetry measurements also consider the differences in laser energy between both intra- and inter-day experiments. A few approaches have been used to measure a radical dose, including using adenine as a dosimeter with UV absorption detection (96), derivatized phenylalanine with isotope dilution GC/MS detection (97), and a reporter peptide that does not require additional detection (98). An adoption of one dosimetry method across all FPOP labs would better standardize the method and make it more widely applicable. The method would also benefit from a robust data analysis platform for both increasing sequence coverage of modified residues and quantitation. The multiple modification types that result from FPOP increase the complexity of identification of modified residues, which is imperative for residue-level structural information. Currently, in many proteomics-based labs and centers, tools such as high-resolution MS hybrid instruments, nanoflow chromatography, automated LC-MS peak detection and alignment software, and Mascot error-tolerant search capabilities can be utilized in combination with each other to enhance the depth of information obtained. The methodology can be adapted to target any protein-footprinting

strategy that imparts stable covalent modifications, and it is well-suited to hydroxyl radical footprinting studies (70). An accessible standard data analysis platform would be beneficial for the use of this method. Although this issue is being addressed, this has not halted the growth and utilization of the technique in even more complex systems.

Recently, an appealing advancement of FPOP points to its potential in studying protein systems in the native cellular environment and in an animal model for human disease. Because of the impact of macromolecular crowding on protein interactions, it is vital to study proteins in their native cellular environment. FPOP has been further extended for *in-cell* analysis to gain structural information across the proteome. *In-cell* FPOP (IC-FPOP) can provide insight into ligand-induced structural changes or conformational changes accompanying protein complex formation, all within the cellular context. Hydrogen peroxide readily crosses cellular membranes, and IC-FPOP has been successfully applied to Vero cells to oxidatively modify several proteins within the cell (24). Proteins can be modified in various organelles, including the nucleus and endoplasmic reticulum increasing the utility of the method for studying a wide variety of proteins regardless of their cellular location. Critical to the success of IC-FPOP was the development of a single-cell flow system (Fig. 6) (47). Hydrodynamic focusing drives the cells along in a single file keeping the cells from clumping to ensure equal exposure to laser irradiation. Because the cells have endogenous catalase, the H_2O_2 and cells are infused separately to limit H_2O_2 degradation. The use of the single-cell flow system led to a 13-fold increase in the number of oxidatively-modified proteins without compromising the dynamic range of the method (47). FPOP also currently shows great promise for *in vivo* applications in *Caenorhabditis elegans* (99). These worms are members of the nematode family and have been used extensively as model systems for human diseases such as a cancer, aging, and diabetes (100). *C. elegans* are transparent to laser irradiation and can ingest H_2O_2 as well as take it up through their skin. Espino and Jones (99) have

extended FPOP modification conditions to successfully oxidatively modify hundreds of proteins within various biological systems in these organisms, including the nervous, digestive, and muscular systems. This method, entitled *in vivo* FPOP (IV-FPOP), measures solvent accessibility inside the worm similar to *in vitro* and IC-FPOP.

The potential of IC-FPOP is its use for proteome-wide structural biology. The ability to report on thousands of proteins in a single experiment along with the fast-labeling timescale extends the applicability of FPOP for studying protein conformational and interaction changes in signaling cascades, cellular stress responses, and other biological processes. Because of its ability to provide biophysical information on many proteins across the proteome, IC-FPOP also has potential as a tool for systems biology. In addition, the method also has the capability to study protein folding in the native cellular environment considering the effect of chaperones and other cellular effects. IV-FPOP has the added potential to study conformations and interactions in an animal model for human disease. Using *C. elegans* as a model system for IV-FPOP allows for the study of various disease states, including those in developmental biology because *C. elegans* are widely used for studying development.

Conclusions

This review presents advantages, limitations, and varied applications of the MS-based structural footprinting method FPOP. FPOP provides the unique capability to obtain residue-level resolution data on protein structures and interactions on the microsecond timescale putting in the regime of fast processes such as protein folding and weak interactions with fast off rates. In addition, the method has been extended for in-cell and *in vivo* analysis making it useful for structural studies across the proteome. Additional development of this technique will further advance the method as a key tool in structural MS-based proteomics in the near future.

Acknowledgment—We thank Daniel Deredge for help with editing.

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